Indian Journal of Animal Sciences **94** (4): 375–380, April 2024/Article
https://doi.org/10.56093/ijans.v94i4.129348

Deciphering proteome changes and meat texture of traditional halal slaughtered spent sheep subjected to low-voltage electrical stimulation and ageing

K B NAMRATHA[1], B M NAVEENA[2]✉, NADEEM FAIROZE[1], RITUPARNA BANERJEE[2], KIRAN MOHAN[3] and MUTHUKUMAR MUTHUPALANI[2]

College of Veterinary Sciences, KVAFSU, Bengaluru, Karnataka 500 030 India

Received: 19 October 2022; Accepted: 8 March 2024

ABSTRACT

The present study evaluated the effect of low-voltage electrical stimulation (LVES, 42 V peak, 0.6 Amp, 50 Hz) and ageing in traditional halal slaughtered Nellore crossbred spent-sheep (*Ovis aries*). The LVES accelerated the post-mortem glycolysis with a significant decline in *p*H and shear force values and improvement in water-holding capacity, cooking yield, protein extractability, and an early onset of rigor-mortis relative to non-stimulated control. Two-dimensional gel electrophoresis coupled with tandem mass spectrometry identified myosin regulatory light chain 2, NADH dehydrogenase, myoglobin, and glyceraldehyde-3-phosphate dehydrogenase from traditional halal slaughtered sheep meat. PANTHER analysis of differentially expressed proteins indicated their involvement in structural (50%), catalytic (25%), and binding (25%) activities. The current study provides a novel insight into the contribution of different proteins in orchestrating meat texture from traditional halal slaughtered spent sheep meat subjected to LVES and ageing.

Keywords: Electrical stimulation, Halal slaughter, Mass spectrometry, Meat texture, Sheep, Two-dimensional gel electrophoresis

Rearing sheep for a longer time to achieve heavier body weight or marketing breeding stock results in relatively tough meat with variable quality. Hence, there is a need for the development of a practical method for improving tenderness or reducing variability in the tenderness of meat from old/ spent sheep to increase their retail value and marketability. Among various available techniques, electrical stimulation of carcasses is a simple, low-cost technology and does not involve incorporation of any chemicals or additives to meat. Low voltage electrical stimulation (LVES) has been reported to be less effective for tenderness improvement when compared to high voltage electrical stimulation, yet LVES application is safer and, hence, a more attractive option. Studies on low voltage electrical stimulation and proteome changes are very much limited. The majority of studies on proteome characterization of skeletal muscles are mainly restricted to beef and the literature on sheep proteome is very scanty. Recently, two-dimensional gel electrophoresis and mass spectrometry for comparative proteome analysis of skeletal muscle between Merino and Tsigai lambs were reported (Gulyas *et al*. 2018). In present authors previous study, the variation in meat quality between sheep that were slaughtered as per the traditional halal method without stunning versus slaughter with electrical stunning was demonstrated and Peroxiredoxin-6, a key potential marker of tenderness in traditional halal slaughtered sheep meat was identified (Kiran *et al*. 2019). Religious slaughter, especially traditional halal slaughter is still predominant in several South, South-west, and South-eastern Asian countries. However, published research papers on the application of proteomic tools to identify the proteins and understand the tenderness variability in traditional halal slaughtered sheep meat and low voltage ES are not available. Hence, the objective of the present study was to investigate the textural changes and to identify important proteins associated with the tenderness of halal slaughtered spent sheep meat subjected to low voltage ES and ageing.

Present address: [1]College of Veterinary Sciences, KVAFSU, Bengaluru, Karnataka. [2]ICAR-National Meat Research Institute, Chengicherla, Boduppal Post, Hyderabad. [3]College of Veterinary Sciences, KVAFSU, Bidar, Karnataka. ✉Corresponding author email: Naveena.BM@icar.gov.in

MATERIALS AND METHODS

Electrical stimulation of sheep carcasses and sampling: Twenty, Nellore crossbred male sheep (*Ovis aries*) around two years age, weighing more than 30 kg were randomly selected from different villages around Hyderabad in Telangana state. Animals were slaughtered according to the traditional Halal method followed in India without prior stunning. Approval was obtained from the Institutional Animal Ethics Committee of ICAR-National Research Centre on Meat, Hyderabad (Approval No. 005/NRCM/IAEC-3). After dressing, carcasses were split into two

halves using an electric carcass splitting saw (FREUD, Model: ST40-13, Schlze-Delitzsch-Str, 38 D-33100 Paderborn, Germany). While splitting the carcasses, one half with the spinal cord was allocated as treatment and subjected to electrical stimulation (ES) within 30 min of exsanguination using electronic stimulation equipment (FREUD, Model: STIM-E512, Schlze-Delitzsch-Str, 38 D-33100 Paderborn, Germany), while the other half from each carcass was used as control (NS). The stimulation was applied by placing the electrode clip in the neck and back (towards the anus) region along the spinal cord for 1 min using a fixed output of 42 V peak (0.6 Amp), 50Hz frequency. The carcass halves were stimulated in a 30 s cycle (2 times) with a 15 s gap in between.

Immediately after electrical stimulation (ES), a small portion (50 g) of *M. Longissimus lumborum* muscle between the fourth and sixth lumbar vertebrae was excised from both control and ES carcass halves and subjected to one h analysis. After 1 h of sampling, the carcasses were chilled at 4±1 ºC in a chiller room for 24 h. At 24 h post-mortem, *M. Longissimus thoracis et lumborum* muscles were removed from each side of the carcass and fabricated into five cm steaks and randomly assigned for ageing at refrigeration temperature for 48 and 72 h. The steaks were subjected to proteomic characterization, physicochemical and textural analysis, Warner–Bratzler shear force (WBSF), and cooking yield. Therefore, the present study includes two treatments (Control and ES), and four sampling times (1 h, 24 h, 48 h, and 72 h) on 20 animals (biological replicates).

Evaluation of physico-chemical quality and textural changes: The *p*H of the meat sample was determined with a digital *p*H meter (Thermo Orion, Model 420A+, USA). The R-value was estimated as per the method described by Honikel and Fischer (1977). The water holding capacity (WHC) was determined as per the method of Kiran *et al*. (2015). Sarcoplasmic proteins and myofibrillar protein extractability were calculated as per the procedures of Joo *et al*. (1999). The per cent cooking yield was estimated by dividing raw weight with cooked weight and multiplying with 100. The Warner-Bratzler shear force (WBSF) of the samples was measured using Texturometer (Tinius Olsen, Model H1KF, 6 Perrywood Business Park, Redhill, RH1 5DZ, England) and was recorded in Newton (N). Fragmentation index values were determined by the procedure outlined by Kadim *et al*. (2009). Collagen content was estimated according to Naveena *et al*. (2004). Collagen solubility was determined by estimating collagen content in raw and cooked meat as described by the method of Dransfield *et al*. (1983).

Protein extraction, purification and two-dimensional gel electrophoresis (2DE): Total muscle protein was extracted and purified as described by Naveena *et al*. (2018). The protein quantification was performed using a 2D quant kit (GE Healthcare, Amersham, Uppsala, Sweden). The first dimension iso-electric focusing (IEF) was carried out in an Ettan IPGPhor3 (GE Healthcare, Uppsala, Sweeden) gel apparatus. Focused IPG strips were equilibrated and the proteins were separated in the second dimension with the SE 600 Ruby apparatus at 100 V with 60 mA/gel. The gels were stained with colloidal Coomassie blue for 3 h followed by overnight destaining. The gels were scanned on an Image Scanner III using labscan 6.0 software, followed by spot detection and quantification with Image Master Platinum7.0 software (GE Healthcare, Uppsala, Sweeden). In-gel digestion of the proteins separated by 2DE was performed as per standard protocols (Shevchenko *et al*. 2006).

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/TOF MS) and bioinformatics analysis: The peptide mixture was dissolved in 0.5 µL of α-Cyano-4-hydroxycinnamic acid (CHCA) matrix solution (5 mg/mL CHCA in 50% acetonitrile/0.1% Trifluoroacetic acid) and spotted onto a freshly cleaned MALDI target plate. After air drying, the crystallized spots were analyzed using a 4800 MALDI-TOF/TOF mass spectrometer (AB Sciex, Framingham, MA) linked to 4000 series explorer software (version 3.5.3). The GPSTM Explorer software version 3.6 (AB Sciex) was used to search the combined MS and MS/MS peak lists. Protein identification was performed by MS/MS ion search using MASCOT version 2.1 (http://www.matrixscience.com) search engine against the Swiss-Prot database. In order to understand the biological process, pathways, and networks, we utilized PANTHER, to generate maximum information from the identified proteins. The differentially expressed proteins were subjected to PANTHER classification system; version 11.1 (http://www.pantherdb.org/).

Statistical analysis: The experimental design was a randomized block design. Fixed effects including ES treatment, ageing time and their interaction, and random effects including animal by ES treatment were analysed using SPSS (SPSS version 13.0 for windows; SPSS, Chicago, IL, USA) and differences among mean values were obtained by Duncan's multiple range tests. Duplicate subsamples used for all the parameters were averaged for statistical analysis. Significance was defined at a level of $P<0.05$. The results were expressed as the least square mean values of three independent replications (n=3) except for Warner-Bratzler shear force, wherein 15 replications were used (n=15).

RESULTS AND DISCUSSION

Physico-chemical characteristics: The initial *p*H (at 1 h) in control and electrically stimulated (ES) sheep meat was 6.29 and 6.15, respectively. During the ageing period, the *p*H reduced significantly ($P<0.05$) for both control and ES samples reaching an ultimate *p*H of 5.71 and 5.55 after 72 h post-mortem, respectively. Low voltage electrical stimulation (LVES) accelerated the *p*H decline resulting in the *p*H of stimulated muscle to become significantly ($P<0.05$) lower than the non-stimulated upto 72 h of post-mortem ageing period. The significant difference in *p*H values of control and electrically stimulated

(ES) sheep meat samples were in agreement with Kadim *et al*. (2009). A progressive increase in R-value was noticed in both ES and control samples. During the rigor onset, depletion of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and further to inosine monophosphate (IMP) occurs, which is incapacitated to provide the energy needed for muscle contraction. The R-value was higher in all the ES meat than in control samples indicating rapid breakdown of ATP and quicker onset of rigor-mortis in ES samples. A significantly (P<0.05) higher R-value in ES samples compared to control meat samples during port-mortem periods in our study revealed that ES accelerates the rigor by full depletion of ATP to IMP. Till 48 h post-mortem, an increase in WHC was observed and then WHC decreased in both ES and control samples (Table 1). A significantly higher (P<0.05) WHC in ES meat samples relative to control samples was observed in the current study which was in accordance with the findings of Li *et al*. (2006). A significant (P<0.05) difference in sarcoplasmic protein extractability between control and treatment was observed at all ageing periods. There was a progressive increase and highly significant (P<0.05) difference in myofibrillar protein extractability and total protein extractability between ES and control samples and also between storage periods. The increase in protein extractability or solubility might have also resulted in increased WHC in ES samples. In the current study higher cooking yield was recorded in ES samples compared to that of control (72.17 % vs 68.10 %). The higher cooking yield in ES meat might be attributed to higher WHC and the water held by meat proteins increased the cooking yield.

Effect on meat texture: The collagen content in sheep meat ranged from 4.088 to 4.340 mg/g of muscle tissue and did not differ (P>0.05) between control and ES samples. There was no significant (P>0.05) increase in collagen solubility in ES samples compared to control during post-mortem ageing. A progressive increase in myofibrillar fragmentation index (MFI) values and significant (P<0.05) difference was observed between control and ES samples and also between post-mortem periods. A significantly (P<0.05) higher myofibrillar fragmentation index (MFI) value was observed in ES samples due to disintegration or physical disruption of myofibrillar ultrastructure and inter-myofibrillar linkages. The shear force values were decreased in both control and ES samples with an increase in the post-mortem ageing. The WBSF values for ES samples were significantly (P<0.05) lower than control samples at all post-mortem periods. In our study, the decrease in shear-force value in electrically stimulated samples was in accordance with the findings of Kadim *et al.* (2009) in lamb carcass.

Effect on proteome changes: The Coomassie-stained 2DE gels from control and ES samples at 24 h post-mortem were shown in Fig. 1A and 1B, respectively. Gels were produced in triplicates and their images were grouped into control and ES. The intensity and area of individual spots were analyzed for comparative image analysis. The class analysis table by ANOVA of control and ES samples indicated 47 spots that had protein spot expression of 1.5-fold or more between them. Among them, significantly (P<0.05) differentially expressed spots as indicated in Fig. 1A and 1B were subjected to mass spectrometry analysis (MALDI-TOF/TOF MS) for further identification. Important proteins identified by the database as per their protein scores are listed in Table 2. Differentially expressed spots were attributed to known proteins based on their molecular weight, isoelectric point, molecular characterization, and functions using the known protein databases. Similar to our findings, Gulyas *et al*. (2018) also observed 327 protein spots in 2DE gels from musculus longissimus dorsi of Merino and Tsigai breeds and found 14 protein spots that showed significant differences in their intensity (P<0.05) between breeds. Variations in the expression of proteins and enzymes were observed between ES and control meat samples. The role of a few important proteins identified

Table 1. Physico-chemical properties and textural changes in control and electrically stimulated (ES) sheep meat

Parameter	Treatment	Post-mortem ageing period (h)			
		0	24	48	72
Water-holding capacity (%)	Control	18.67 ± 0.67^{aX}	21.53 ± 0.33^{cX}	23.33 ± 0.33^{dX}	19.67 ± 1.20^{bX}
	ES	23.33 ± 0.67^{bY}	25.00 ± 1.00^{cY}	29.33 ± 0.88^{dY}	22.33 ± 2.00^{aX}
Total protein extractability (mg/g protein)	Control	187.67 ± 1.45^{aX}	210.67 ± 1.76^{bX}	244.33 ± 1.23^{cX}	324.00 ± 2.03^{dX}
	ES	226.67 ± 1.20^{aY}	255.33 ± 2.60^{bY}	301.33 ± 1.86^{cY}	293.33 ± 2.03^{dY}
Sarcoplasmic protein extractability (mg/g protein)	Control	46.00 ± 1.00^{aX}	66.67 ± 1.67^{bX}	105.00 ± 2.89^{cX}	155.00 ± 2.89^{dX}
	ES	67.67 ± 1.45^{aY}	94.00 ± 1.00^{bY}	129.00 ± 1.73^{cY}	163.33 ± 1.76^{dX}
Myofibrillar protein extractability (mg/g protein)	Control	141.6 ± 2.40^{aX}	144.00 ± 0.58^{aX}	139.33 ± 2.96^{aX}	169.00 ± 2.20^{bX}
	ES	159.0 ± 1.15^{bY}	161.33 ± 2.53^{bY}	172.33 ± 0.67^{cY}	130.00 ± 2.51^{aY}
Myofibrillar fragmentation Index	Control	64.60 ± 0.98^{aX}	67.90 ± 0.20^{bX}	70.33 ± 0.34^{cX}	75.33 ± 0.44^{dX}
	ES	70.86 ± 0.20^{aY}	74.63 ± 0.46^{bY}	79.46 ± 0.21^{cY}	82.76 ± 0.32^{dY}
Warner-Bratzler shear force (N)*	Control	36.42 ± 0.82^{aX}	29.950 ± 0.35^{bX}	24.922 ± 0.35^{cX}	20.125 ± 0.36^{dX}
	ES	24.55 ± 0.55^{aY}	21.344 ± 0.18^{bY}	19.118 ± 0.31^{cY}	16.409 ± 0.36^{dY}

Values are Mean±SE of 3 replications (n=3) and n=15* for Warner-Bratzler Shear force; Significance level at P<0.05; a-d, indicates significance between post-mortem ageing (column-wise); X,Y, indicates significance between treatments (row-wise).

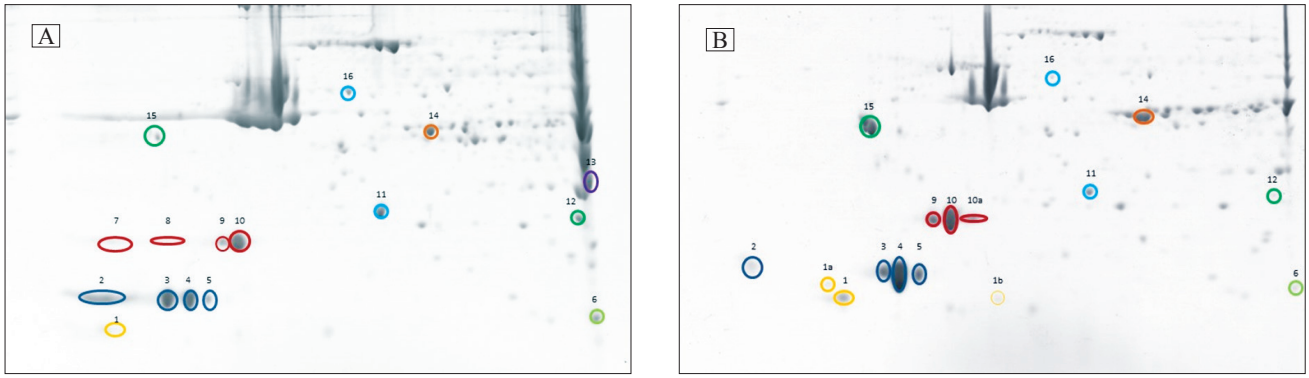

Fig. 1. Representative 2-DE image indicating differentially expressed proteins after 24 h chilling in control (A) and electrically stimulated sheep meat (B). Spots that significantly (P<0.05) changed in expression between control and ES meat proteome are indicated by numbers.

in the study which have greater relevance to meat quality and texture (Table 2) are discussed. The NADH dehydrogenase is involved in the electron transport chain for the generation of ATP. A similar potential marker was detected in traditional halal slaughtered sheep meat (Kiran *et al*. 2019), which positively correlates with the WBSF results. Glyceraldehyde-3-phosphate dehydrogenase, a widely known glycolytic enzyme is involved in regulating proteins, and supportive metabolic activities consistent with tenderness characteristics (Kiran *et al*. 2019). Tropomyosin expressed in fast skeletal muscle, binds to actin filaments and plays a major role in the calcium-dependent regulation of vertebrate skeletal muscle contraction in association with Troponin-T. The tropomyosin, ubiquinone, calcium-binding protein, calcium channel protein, and ATP synthase that might be responsible for the tenderization of sheep meat due to electrical stimulation were identified (Ouali *et al*. 2013).

Using 'Protein Analysis Through Evolutionary Relationships' (PANTHER) analysis, the identified proteins were classified based on their molecular function and biological process (Fig. 2A and 2B). About 50% of identified proteins belonged to structural activity whereas, 25% of proteins were associated with catalytic and 25% of the remaining proteins were involved with binding activity. The proteins identified in the present study belong to various classes, viz. nucleic acid binding, transporter, cytoskeletal, calcium-binding, and membrane traffic

Table 2. List of differentially expressed proteins identified using MALDI-TOF/TOF MS analysis of protein spots from 2-DE gels between control and electrically stimulated (ES) meat samples

Spot no.	Accession no.	Protein identified	Mass	Protein score	Molecular function	Biological process
C-1	ADT3	ADP/ATP translocase 3	33084	33	ADP antiporter activity	Apoptotic process, Regulation of mitochondrial membrane permeability
C-2	MLRS	Myosin regulatory light chain 2, skeletal muscle isoform type 2	19128	84	Calcium ion binding	-
C-7	NDUA7	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 7	12615	46	NADH dehydrogenase	ATP synthesis coupled electron transport
C-8	COX5	Cytochrome c oxidase subunit 5B, mitochondrial	14002	30	Cytochrome-c oxidase activity	Mitochondrial ATP synthesis coupled proton transport
C-9	RGS6	Regulator of G-protein signaling 6	7526	32	GTPase activator activity	Negative regulation of signal transduction
C-16	CXCL5	C-X-C motif chemokine 5	12250	28	Chemokine activity	Cell-cell signaling
T-1	NDUB1	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 1	6962	35	NADH dehydrogenase activity	Electron transport
T-1[a]	G3P	Glyceraldehyde-3-phosphate dehydrogenase	36141	33	Microtubule binding	Glucose metabolic process
T-6	MYG	Myoglobin	17275	24	Heme binding	Oxygen transport
T-6	CALCA	Calcitonin gene-related peptide 1	14110	24	Calcitonin receptor binding	Aging
T-15	TPM1	Tropomyosin alpha-1 chain	32744	97	actin filament binding	Actin filament organization

C, Control; T, ES Samples.

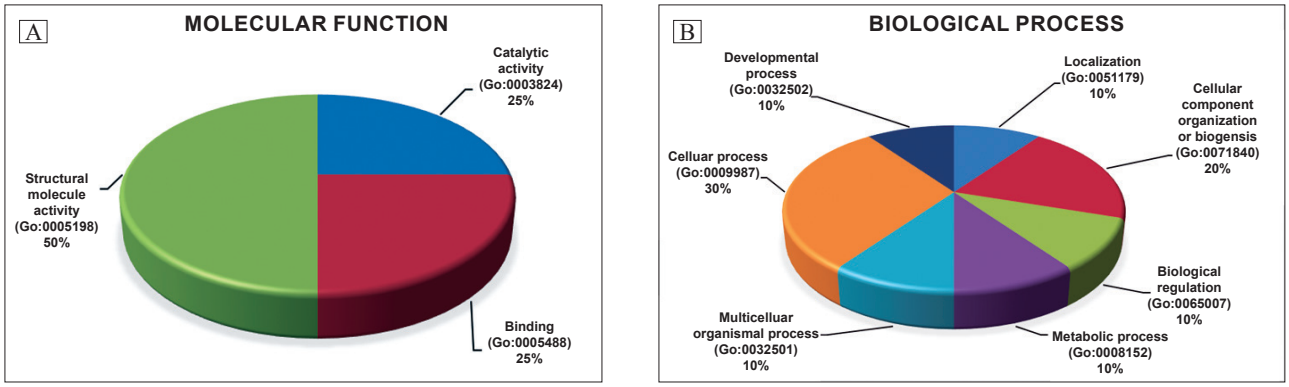

Fig. 2. Illustration of (A) Molecular function and (B) Biological process for control vs electrically stimulated (ES) muscle proteome using PANTHER analysis of the differentially expressed protein dataset.

proteins. Identification of proteins that are associated with a metabolic process in the current study indicates the beneficial effect of ES on the glycolytic pathway and associated changes due to *p*H reduction. Contreras-Castillo *et al*. (2016) studied the effect of electrical stimulation on post-mortem myofibrillar protein degradation and small heat shock protein kinetics in bull beef and observed that HSP20 concentrations in ES muscles were significantly higher compared with NS muscles early post-mortem and at later ageing time periods. Several proteins identified in the current study are also reported by Kiran *et al*. (2019) in Halal slaughtered sheep meat that are associated with meat texture.

It can be concluded that the low voltage electrical stimulation (LVES) significantly accelerated the post-mortem glycolysis, as evident from rapid glycogen depletion, *p*H decline, and the onset and completion of rigor mortis in stimulated carcass compared to control. The LVES caused a significant improvement in sheep meat tenderness which was evident in decreased WBSF values and improved sensory scores. The proteomic analysis using 2DE, MS identification, and PANTHER analysis revealed the involvement of structural proteins and their role in accelerated proteolysis due to ES of halal slaughtered sheep. Present findings provide a better understanding of the biochemical processes taking place as a result of LVES during ageing of halal slaughtered sheep meat. Hence, LVES may be used as an effective post-slaughter intervention for optimizing the meat quality from halal slaughtered spent sheep.

ACKNOWLEDGEMENTS

This study was funded by the Indian Council of Agricultural Research LBSYSA Grant (Edn./34/4/2015-HRD). The authors wish to thank Director, ICAR-National Meat Research Institute for providing the necessary facilities to undertake this work.

REFERENCES

Contreras-Castillo C J, Lomiwes D, Wuc B G, Frost D and Farouk C M. 2016. The effect of electrical stimulation on post mortem myofibrillar protein degradation and small heat shock protein kinetics in bull beef. *Meat Science* **113**: 65–72.

Dransfield E, Casey J C, Boccard R, Touraille C, Buchter L, Hood D E, Joseph R L, Schon I, Casteels M, Casentino E and Thimbergen B J. 1983. Comparison of chemical composition of meat determined at eight laboratories. *Meat Science* **8**: 79–85.

Gulyas G, Pohoczky K, Csosz E, Simon A, Javor A and Czegledi L. 2018. Comparative proteome analysis of skeletal muscle between Merino and Tsigai lambs. *Small Ruminant Research* **158**: 35–41.

Honikel K O and Fischer C. 1977. A rapid method for detection of PSE and DFD in porcine muscle. *Journal of Food Science* **42**: 1633–36.

Joo S T, Kauffman R G, Kim B C and Park G B. 1999. The relationship of sarcoplasmic and myofibrillar protein solubility to colour and water holding capacity in porcine longissimus muscle. *Meat Science* **52**: 291–97.

Kadim I T, Mahgoub O, Al-Marzooqi W, Khalaf S, Al-Sinawi S S H and Al-Amri I S. 2009. Effects of transportation during the hot season and low voltage electrical stimulation on histochemical and meat quality characteristics of sheep longissimus muscle. *Livestock Science* **126**: 154–61.

Kiran M, Naveena B M, Reddy K S, Shashikumar M, Reddy V R, Kulkarni V V, Rapole S and More T H. 2015. Muscle-specific variation in buffalo (*Bubalus bubalis*) meat texture: Biochemical, ultrastructural and proteome characterization. *Journal of Texture Studies* **46**: 254–61.

Kiran M, Naveena B M, Smruthirekha M, Baswa Reddy P, Rituparna B, Praveen Kumar Y, Venkatesh Ch and Rapole S. 2019. Traditional halal slaughter without stunning versus slaughter with electrical stunning of sheep (*Ovis aries*). *Meat Science* **148**: 127–36.

Li C B, Chen Y J, Xu X L, Huang M, Hu T J and Zhou G H. 2006. Effects of low-voltage electrical stimulation and rapid chilling on meat quality characteristics of Chinese Yellow crossbred bulls. *Meat Science* **72**: 9–17.

Naveena B M, Mendiratta S K and Anjaneyulu A S R. 2004. Tenderization of buffalo meat using plant protease from *Cucumis trigonus Roxb* (Kachri) and *Zingiber officinale roscoe* (Ginger rhizome). *Meat Science* **68**: 363–69.

Naveena B M, Jagadeesh D S, Kamuni V, Muthukumar M, Kulkarni V V, Kiran M and Rapole S. 2018. In-gel and OFFGEL-based proteomic approach for authentication of meat species from minced meat and meat products. *Journal of the Science of Food and Agriculture* **98**(3): 1188–196.

Ouali A, Gagaoua M, Boudida Y, Becila S, Boudjellal A, Herrera-Mendez C H and Sentandreu M A. 2013. Biomarkers of meat tenderness, Present knowledge and perspectives in regards to our current understanding of the mechanisms involved. *Meat Science* **95**: 854–70.

Shevchenko A, Tomas H, Havlis J, Olsen J V and Mann M. 2006. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nature Protocols* **1**: 2856–860.